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Common Origin of Human T-Lymphotropic Virus Type-I From Iran, Kuwait, Israel, and La Réunion Island

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We found previously that Kuwaiti HTLV-I isolates had two nucleotide substitutions in the most frequently sequenced regions of HTLV-I genome, namely: T → C 4783 in the *pol* and T → C 6569 the *env* genes. These substitutions were observed rarely in other HTLV-I isolates and seemed to be good markers of the HTLV-I lineage, the ''epicentre'' of which was located in Mashhad, Iran. To test this hypothesis we sequenced the fragments of HTLV-I genome including sites 4783 and 6569 from seven isolates obtained from the Iranians either living in (five isolates) or originating from (two isolates) Mashhad. RFLP-based tests were also designed and used for typing of the substitutions. All seven isolates were positive for T → C 4783 and six, from which *env* fragment was amplifiable, were also positive for T → C 6569. It is highly probable that all the isolates from Mashhadi Jews belong to the same HTLV-I lineage, although they were not typed yet for the presence of T → C 6569 substitution. Only 2 ''non-Middle Eastern'' HTLV-1, both from La Réunion Island were positive for both of the substitutions. Another possible member of Mashhadi lineage of HTLV-I is one isolate from southern India and two isolates from the American Indians, British Columbia, Canada. The determination of the T → C 4783 and T → C 6569 markers in HTLV-I isolates of different geographical/ethnic origin may be useful for the reconstruction of the routes of HTLV-I spread from the Middle East and/or Indian subcontinent to other regions of the world and, possibly, for gaining insights into the origin of HTLV-I in Asia. ***J. Med. Virol. 52:77–82, 1997.***

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INTRODUCTION

Human T-lymphotropic virus type 1 virus (HTLV-I) is a human retrovirus considered as the etiological agent of adult T-cell leukemia/lymphoma (ATLL) and a form of neuromyelopathy referred to as TSP/HAM—tropical spastic paraparesis (TSP) or HTLV-associated myelopathy (HAM) [Yoshida et al., 1984; Gessain et al., 1985]. The association of HTLV-I with some other human diseases has also been described [for review: see Gessaine, 1996]. HTLV-I infection is usually asymptomatic. It is estimated that ATLL or TSP/HAM develop in only 2.5–4% HTLV-I carriers with a very long incubation period (10 to 50 years for ATLL).

The prevalence of HTLV-I infection varies significantly between different regions of the world. The "classical" HTLV-I high prevalence regions are southwest of Japan, the Carribean islands, and some areas of Central and Western Africa [Hinuma et al., 1981; Blattner et al., 1982; de-Thé et al., 1989]. "Pockets" of HTLV-I infection have also been found in other regions of the world. Although HTLV-I from the Middle East was described for the first time as early as 1983 [Popovic et al., 1983], this region was considered as having very low prevalence of this infection and free from the high prevalence "HTLV-I pockets" up until the beginning of the 1990s. In 1990, a cluster of HTLV-I infection was found for the first time in the Middle East, namely, in Israel [Meytes et al., 1990]. The Israelis infected with HTLV-I were shown to be almost exclusively immigrants from the northeast of Iran, the so called Mashhadi Jews [Meytes et al., 1990; Sidi et al., 1990; Achiron et al., 1993]. Later, it was also shown that the high prevalence of HTLV-I infection is also characteristic

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TABLE I. List of HTLV-I Isolates Typed for the Presence of T → C 4783 and T → C 6569 by RFLP Analysis and the Sequencing of Corresponding *pol* and *env* Genes Fragments

No.	Isolate	Diagnosis	Age/sex	Origin	POL	ENV
1.	IRN-1	ATL	Unknown	Mashhad	+[a]/+[b]	+[c]/+[d]
2.	IRN-2	ATL	Unknown	Mashhad	+/+	+/+
3.	IRN-4	Unknown	Unknown	Mashhad	+/+	+/+
4.	IRN-5	TSP/HAM	Unknown	Mashhad	+/+	+/+
5.	IRN-7	Unknown	Unknown	Mashhad	+/+	na[e]
6.	SAS	ATL	60y/f	Mashhad[f]	+/+	+/+
7.	SHA	Asympt.	67y/f	Mashhad[f]	+/+	+/+
8.	Abl.A	Asympt.	67y/m	La Réunion	+/+	+[g]/+
9.	Abl.M	Asympt.	79y/f	La Réunion	+/+	+[g]/+
10.	C91-PL	PTCL[h]	29y/f	USA	–/–	–/–

[a]Sequence of 213 bp *pol* gene fragment, C (+) or T (–) at position 4783.
[b]Result of RFLP test for T → C 4783 positive (+) or negative (–).
[c]Sequence of 150 bp *env* gene fragment, C (+) or T (–) at position 6569.
[d]Result of RFLP test for T → C 6569 positive (+) or negative (–).
[e]*env* gene fragment was not amplifiable (na).
[f]Iranian immigrants in France originating from Mashhad [Gabarre et al., 1993].
[g]Sequence of *env* gene fragment was reported previously [Mahieux et al., 1994].
[h]Peripheral T-cell lymphoma-PTCL [Popvic et al., 1983].

of the Muslim population of Mashhad [Kitze et al., 1992; Gabarre et al., 1993; Vidal et al., 1994; Puccioni-Sohler et al., 1995; Safai et al., 1996, Farid et al., 1995]. Cases of HTLV-I infection and HTLV-I-associated diseases have also been described in Iraq [Denic et al., 1990] and, recently, in Kuwait [Voevodin et al., 1995; Farah et al., 1996; Al-Mufti et al., 1996].

In a previous study [Voevodin et al., 1995], we found that Kuwaiti HTLV-I isolates had two nucleotide substitutions in the most frequently sequenced regions of HTLV-I genome, namely, in the 140 bp fragment of the *pol* gene [Sherman et al., 1992] and in the 522 bp fragment of the *env* gene [Gessain et al., 1992]. These substitutions (T → C 4783 and T → C 6569; numbering of nucleotides as in Los Alamos retrovirus database) were rarely observed in other HTLV-I isolates of the same, cosmopolitan subtype and seemed to be good markers of the Middle Eastern HTLV-I lineage. However, at that time very few HTLV-I isolates were sequenced in both of the genomic regions and none of the latter (except Kuwaiti isolates) had both T → C 4783 and T → C 6569 or originated in the Middle East. Those few isolates which were positive for either T → C 4783 or T → C 6569 were not sequenced in both *pol* and *env* genomic regions.

We now report 15 new genomic sequences of HTLV-I isolates from Iran and La Réunion Island. All these isolates, as well as the previously described Kuwaiti HTLV-I isolates, have both T → C 4783 and T → C 6569 substitutions, a feature that seems to be quite specific for the Middle Eastern (Mashhadi) lineage of the HTLV-I. The short report of these results has been presented at the XVIII International Association for Comparative Research on Leukemia and Related Diseases Symposium, October, 1995.

MATERIALS AND METHODS

DNA Samples

Information on HTLV-I positive subjects investigated in this study is shown in Table I. DNA was extracted from the peripheral blood cells as described previously [Gessain et al., 1992].

PCR Amplification

The fragments of *pol* and *env* gene were amplified by nested and semi-nested PCR using primers described previously [Voevodin et al., 1995]. The 253 bp *pol* gene fragment sequenced was amplified by nested PCR using primers AV-22/AV-23 at the first step and primers AV-20/AV-21 at the second step. The 379 bp *pol* gene fragment used for RFLP typing of T → C 4783 substitution was amplified by semi-nested PCR using primers AV-22/AV-23 at the first step and primers AV-22/AV-21 at the second step of this test. The 196 bp *env* gene fragment used for both sequencing and RFLP typing of T → C 6569 substitution was amplified by semi-nested PCR using primers AV-1/AV-2 at the first step and primers AV/14/AV-2 at the second step of this test. All PCR tests were carried out using standard PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl, 1.5 mM $MgCl_2$), 50 μM of deoxynucleoside triphosphates, 10 pmol of each primer and approximately 500 ng of genomic DNA in a total volume of 25 μl. Thermocycling was undertaken in Perkin-Elmer system 9600. The cycling protocol used was 94°C, 55°C, and 72°C, 30 sec at each temperature, 30 cycles. Standard precautions to prevent carry-over, were strictly followed [Kwock and Highushi, 1989]. The detection of the amplimers was carried out by agarose minigel electrophoresis and staining with ethidium bromide.

DNA Sequencing

PCR amplified fragments of *env* and *pol* genes were sequenced directly using ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS, and the Applied Biosystems model 373 automatic DNA sequencer according to the manufacturer's (Applied Biosystems/Perkin Elmer Foster City, USA) protocols. To minimize artifacts due to *Taq* polymerase mistakes, samples were prepared for sequencing as a

TABLE II. Typing for of T → C 4783 and T → C 6569 Substitutions in Cosmopolitan HTLV-I Genome by RFLP Analysis-Expected Fragments

	POL
Undigested:	379 bp
*Hinf*I digest	
4783T:	323 bp, 56 bp
4783C:	171 bp, 152 bp, 56bp
	ENV
Undigested:	206 bp
*Fok*I digest	
6559T:	166 bp, 40 bp
6559C:	149 bp, 40 bp, 17 bp

pool of ten separate PCR amplifications with the same target DNA and primers.

DNA Sequence Characterization and Phylogenetic Analysis

DNA sequences were aligned, compared, and analyzed for the distribution of restriction endonuclease sites by using DNASIS program (Hitachi Bris Bane, USA).

Restriction Fragment Length Polymorphism (RFLP) Tests for T → C 4783 and T → C 6569 Substitutions

PCR amplified *pol* and *env* gene fragments were purified using MicroSpin S-200 HR Columns (Pharmacia Biotech Uppsala, Sweden). The *pol* fragment was digested with the restriction endonuclease *Hinf I* (Pharmacia Biotech Uppsala, Sweden) and the *env* fragment was digested with the restriction endonuclease *Fok I* (Amersham/USB Little Chalfont, England), according to the instructions of the manufacturers. The digests were analyzed by agarose minigel electrophoresis/ethidium bromide staining. The information on the size of the fragments generated in the presence or absence of T → C 4783 and T → C 6569 substitutions is shown in Table II.

RESULTS

Seven HTLV-I isolates from Mashhad, Iran (five from the people born and living there and two from Iranian immigrants who were born and lived in Mashhad before moving to France) have been characterized. The 379 bp and 253 bp fragments of the *pol* gene were successfully amplified from all of these isolates by semi-nested and nested PCR, respectively. The 196 bp fragment of *env* gene was amplified from six isolates by semi-nested PCR. In one case (isolate IRN-7), amplification was not achieved. In addition to the Iranian/Mashhadi HTLV-I isolates, three more isolates have been characterized, two of these from La Réunion Island [Mahieux et al., 1994] and one from the USA [Popovic et al., 1983]. The La Réunion isolates (Abl.A and Abl.M) have been sequenced only in the *pol* gene region, because their *env* gene sequences have been reported [Mahieux et al., 1994]. The presence of T → C 6569 substitution in the both of them was the reason why these isolates were chosen for characterization regarding the presence of T → C 4783 substitution in the *pol* gene. The American isolate was used as a control in amplification and DNA sequencing experiments. All isolates sequenced in this study, except for the American isolate, C91-PL, were positive for both T → C 4783 and T → C 6569 substitutions on the *pol* and *env* genes, respectively (Table I, columns 5 and 6, nominator). All the T → C 4783 and T → C 6569 positive sequences were identical to the homologous sequences of Kuwaiti HTLV-I isolates reported earlier (GenBank Accession Numbers: L42254 for the *pol* sequences and L42221 for the *env* sequences).

The presence of these substitutions has been confirmed independently using restriction fragment length polymorphism (RFLP) analysis (Table I, column 5 and 6, denominator). Restriction enzymes *Hinf I* and *Fok I* have been used for typing of the T → C 4783 and T → C 6569 substitutions, respectively. The fragments generated by these enzymes in the presence or absence of T → C 4783 and T → C 6569 substitutions are shown in Table II. Representative results of RFLP typing are presented in Figure 1. As was mentioned above, in addition to the HTLV-I isolates characterized in this study, three other Middle Eastern isolates (KUW-1, 2, and 3 from Kuwaitis, most probably originating from southern Iraq) have been shown to contain both the T → C 4783 and T → C 6569 substitutions [Voevodin et al., 1995].

The first of these substitutions was present in all 15 Middle Eastern isolates (three from Kuwait, seven from Iran, and five from Israel) sequenced in this region. Only five of 44 HTLV-I isolates originating from other regions of the world were positive for T → C 4783. Interestingly, three (Abl.A, Abl.M, and CMCH13 from La Réunion Island and India, respectively) quite possibly belong to the same lineage as Iranian/Mashhadi HTLV-I. Two of the others were the isolate CH from the Caribbean and the isolate ZA35593 from Zaire. It should be noted that one of these isolates (CH) is negative for T → C 6569 substitution and thus, it does not have both molecular markers of the "Mashhadi lineage" of cosmopolitan HTLV-I. The second exception (isolate ZA35593), was not sequenced in the relevant *env* gene region and was not available for our study.

The second substitution characteristic for the Middle Eastern HTLV-I (T → C 6569) was found in 11 out of 11 Middle Eastern isolates sequenced in this region of the genome. Six of these sequences (IRN-1, IRN-2, IRN-4, IRN-5, SAS, and SHA) are reported in this paper; five others (KUW-1, KUW-2, KUW-3 [Voevodin et al., 1995], MA7, and SXH9 [Safai et al., 1996]) have been reported previously. Only four out of 73 cosmopolitan HTLV-I isolates from other regions of the world were positive for this substitution: the same two isolates from La Réunion Island (Abl.A and Abl.M) and two

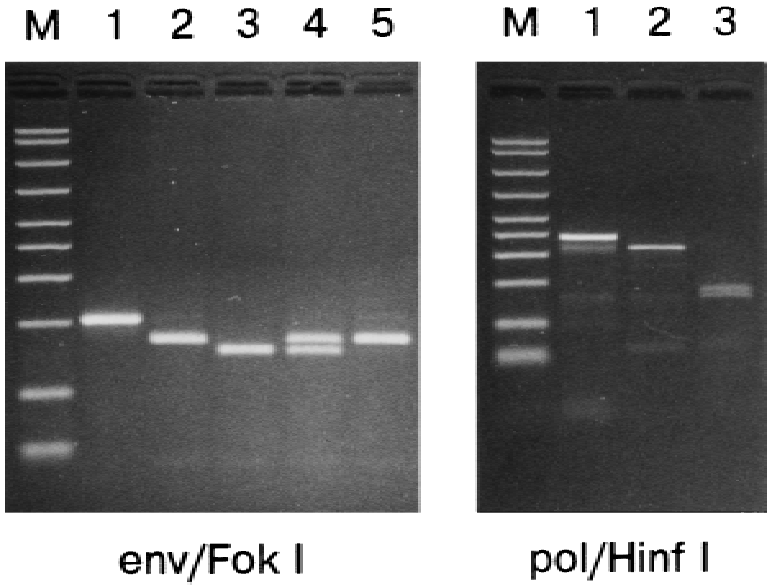

Fig. 1. M, DNA size markers, bottom to top: 50, 100, 200, 300, 400, 500, 700, 1000, 1500, and 2000 bp. env/Fok I, RFLP typing of T → C 6569 substitution. 1–206 bp *env* fragment, undigested. 2,5–206 bp *env* fragment from C91-PL after digestion with *Fok I.* 3–206 bp *env* fragment from IRN-2 after digestion with *Fok I.* 4, Mixture of *Fok I* digested fragments from C91-PL and IRN-2. pol/Hinf I, RFLP typing of T → C 4783 substitution. 1–379 bp *pol* fragment, undigested. 2–379 bp *pol* fragment from C91-PL after digestion with *Hinf I.* 3–379 bp *env* fragment from IRN-2 after digestion with *Hinf I.* Faint bands below 379 bp *pol* fragment and between 100 and 200 bp markers are nonspecific. The presence of these bands did not interfere with the interpretation of RFLP typing of T → C 4783 substitution.

isolates (BCI1-1 and BCI1-2) from American Indians from British Columbia, Canada [Picard et al., 1995].

DISCUSSION

The genome of HTLV-I is remarkably stable [Gessain et al., 1992]. This feature provides a potential, at least in theory, to trace the origin of different HTLV-I isolates and to correlate the patterns of the distribution of different HTLV-I subtypes and lineages with the ancient and modern movements of different human ethnic groups and populations.

Most of the Middle Eastern HTLV-I isolates characterized during the last few years were the isolates from Mashhadi Jews [Achiron et al., 1993; Kilim et al., 1994; Danon et al., 1994; Nerurkar et al., 1995; Yamashita et al., 1995a,b].

This relatively closed ethnic group, before recent immigration to Israel, lived for several hundred years in Mashhad, Iran. It was suggested that HTLV-I accumulated in this group due to its strict isolation from the Muslim population of Mashhad. Obviously, HTLV-I had to be first introduced in this population. But, from where and when? There are two possibilities: at least one of members of the 40 Jewish families which settled in Mashhad in 1740 was HTLV-I positive prior to settling down in Mashhad; and secondly, HTLV-I was introduced in this population after their arrival at Mashhad. It is hardly possible to verify which of these possibilities is true, and they are not mutually exclusive. However, the second possibility could be excluded, if HTLV-I would be absent in the Muslim population of Mashhad. On the other hand, if HTLV-I is present in the Muslim population of Mashhad, its molecular characterization could provide some clues as to the origin of HTLV-I in the Middle East. The first data suggesting that HTLV-I infection is common in Muslims from the Mashhad region were obtained in the studies of Iranian immigrants in several West European countries [Kitze et al., 1992; Gabarre et al., 1993]. It was shown that a surprisingly high proportion of HTLV-I positive cases observed in Germany, France, and the UK comprised of Iranians originating from the Mashhad region. HTLV-I was also described in Iraq, southern region of which had close historical ties with Mashhad; the latter is a holy city for Shiite Muslims and southern Iraq is populated mainly by Shiites. HTLV-I was also described in Kuwait, again in the families originating from southern Iraq and specifically from the city of Najaf which for centuries had close ties with Mashhad [Voevodin et al., 1995; Farah et al., 1996]. Thus, the pattern had been emerging suggesting that a cluster of HTLV-I infection in the Mashhadi Jews is not an isolated one, but just one of the "derivatives" of the same lineage of cosmopolitan HTLV-I which had spread throughout the Middle East from Mashhad. To test this hypothesis the sequences of different Middle Eastern HTLV-I isolates had to be generated and compared.

The additional difficulty was the inability of commonly used phylogenetic analysis to reliably discriminate between different HTLV-I isolates within cosmopolitan A "sub-subtype" to which Mashhadi HTLV-I isolates belong [Voevodin et al., 1995]. To overcome this difficulty we employed a simplistic but, quite informative approach: the identification of the "lineage-specific" combination of nucleotide substitutions i.e., the substitution which alone or in combination are

unique or almost unique for the Mashhadi HTLV-I isolates. Two good candidates for such Mashhadi HTLV-I lineage markers, T → C 4783 in the *pol* gene and T → C 6569 in the *env* gene have been identified [Voevodin et al., 1995].

In this study it was shown both by sequencing and RFLP analysis that these substitutions are present in all seven Iranian HTLV-I isolates studied, five of which were directly from Mashhad. Unfortunately, none of the isolates from Mashhadi Jews have been sequenced in the region including position 6569. However, there are strong reasons to believe that all of them will be found to be positive for T → C 6569. Among the HTLV-I isolates which have been sequenced in the genomic regions including both positions 4783 and 6569, there are only two non-Middle Eastern isolates which are positive for both the T → C 4783 and T → C 6569. Interestingly, both isolates are from inhabitants of La Réunion Island which was a stopover for Indian immigrants, mostly from the southwest coast of Indian subcontinent, the region with extensive historical contacts with the Middle East. Thus, it is quite possible that the Mashhadi lineage of HTLV-I was introduced in this region of India from the Middle East and was then brought to La Réunion Island. This interpretation is supported by the presence of T → C 4783 substitution in one HTLV-I isolate from the Kerala state in the south of India [Nerurkar et al., 1993]. Unfortunately, the Indian isolate is not yet characterized in terms of the presence of the T → C 6569 substitution. The alternative possibilities for the introduction of Mashhadi HTLV-I to India could be relatively recent migration of Jews from Mashhad to India in 18th and 19th centuries [Achiron et al., 1993] or the more ancient introduction with invaders entering the Indian subcontinent from the northwest.

Another surprising link between Middle Eastern lineage of HTLV-I and the isolates from other parts of the world is the presence of T → C 6569 substitution in two isolates (BCI1-1 and BCI1-2) from American Indians from British Columbia, Canada [Picard et al., 1995]. Interestingly, there is very high degree of similarity between the Middle Eastern, La Réunion Island, and British Columbia isolates also in LTR region [Voevodin, unpublished]. A trivial explanation for that can be relatively recent introduction of the Middle Eastern lineage of HTLV-I into British Columbia with the immigrants from Iran, Gulf countries, and India. The more intriguing possibility is the ancient introduction of this lineage of HTLV-I into America with the people which populated America from Asia. If the latter is true Mashhadi lineage of HTLV-I might be the most ancient Asian variant of HTLV-I. It should be emphasized, however, that this hypothesis is highly speculative and many HTLV-I isolates from Asia and the rest of the world must be characterized before any conclusion can be drawn. It is also not clear how far back in time would such reconstruction be possible. However, the task is worth pursuing, especially in the context of clarification of the origin of HTLV-I in Asia.

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